(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 356 739 A1**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 89114207.7

(22) Date of filing: 01.08.89

(51) Int. Cl.5: C12N 15/52 , C12N 15/77 , C12N 1/20 , C12P 13/06 , C12P 13/08 , //(C12N15/52, C12R1:13,1:15)

The microorganism(s) has (have) been deposited with Fermentation Research Institute under number(s) FERM P-10142, FERM P-10140, FERM P-10143 and FERM 10141.

(30) Priority: 03.08.88 JP 194031/88

(43) Date of publication of application: 07.03.90 Bulletin 90/10

(84) Designated Contracting States: DE FR NL

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(54) Recombinant DNA, microorganism carrying said recombinant DNA, and process for producing L-amino acids by the use of said microorganism.

(57) The invention relates to a plasmid of phage capable of autonomic multiplication having the gene for acetohydroxy acid synthase incorporated therein, to a microorganism carrying a plasmid or phage as defined above, a recombinant DNA comprising the gene coding for acetohydroxy acid synthase and a process for producing L-valine, L-leucine or L-isoleucine, which comprises growing a microorganism as defined above and recovering the objective amino acid from the culture liquor.

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Recombinant DNA, Microorganism Carrying Said Recombinant DNA, and Process for Producing L-Amin Acids by the Use of Said Microrganism

This invention relates to recombinant DNA having the gene for acetohydroxy acid synthase incorporated therein, and to a process for producing L-valine, L-leucine or L-isoleucine by the use of a microorganism capable of producing one of these amino acids that carries said recombinant DNA.

[Prior Art]

For the manufacture of L-valine, L-leucine and L-isoleucine by the fermentation process, has been adopted the method in which a wild strain is imparted with the ability of producing one of these amino acids by artificial mutation, because wild strains scarcely produce these amino acids outside the microbial cells.

As the artificial mutants capable of producing L-valine, L-leucine or L-isoleucine, are known those belonging to the genus Brevibacterium, Corynebacterium or Serratia and resistant to antagonist against these amino acids. To be more specific, the method consists in the use of mutants in which acetohydroxy acid synthase (the key enzyme participating in the production of L-valine, L-leucine or L-isoleucine) is less subject to feedback inhibition by its end product (L-valine, L-leucine and/or L-isoleucine) or antagonist thereof.

Such mutants as mentioned above have the problem that only one gene that codes for acetohydroxy acid synthase is involved in each genome, and hence its enzymatic activity is limited even when released from the feedback inhibition. In addition, it is not an easy task to release each strain from the feedback inhibition.

[Problems to be Solved by the Invention]

The object of this invention is to provide a method of artificially controlling or nullifying the metabolic regulation in the L-valine, L-leucine or L-isoleucine biosynthesis system, thereby eliminating the defect of limited number of genes associated with the conventional method, and facilitating the release from feedback inhibition. To put it concretely, the method proposed in this invention is intended to release acetohydroxy acid synthase (the key enzyme common to the biosynthesis of L-valine, L-leucine and L-isoleucine) from feedback inhibition by these amino acids and to increase the number of genes for this enzyme in each genome.

[Means to Solve the Problems]

Intensive studies on the above-mentioned problems have led us to find that the L-valine, L-leucine or L-isoleucine productivity can be enhanced by isolating from a microorganism, preferably of Brevibacterium, a DNA fragment that codes for acetohydroxy acid synthase, incorporating this fragment into a plasmid capable of autonomic multiplication, and introducing this recombinant plasmid into a coryneform glutamate-producing bacterium (e.g., Brevibacterium lactofermentum).

The DNA fragment carrying the gene for acetohydroxy acid synthase can be obtained from the chromosomal DNA of a microorganism of Brevibacterium. Such microorganisms form which a DNA carrying genetic information (e.g., DNA fragment carrying the gene for acetohydroxy acid synthase) can be obtained are hereinafter referred to as DNA donors.

As the DNA donor, microorganisms can be used which show a relatively high activity in the production of L-valine, L-leucine or L-isoleucine. Such microorganisms are known to a person skilled in the art. Preferably, there may be used those mutants which have been imparted with resistance to antagonists of L-valine, L-leucine or L-isoleucine and hence show higher biosynthetic activity for these amino acids or precursors thereof. Preferred examples of such DNA donors are microorganisms of the genus Brevibacterium, more preferably of the species Brevibacterium lactofermentum which have high bioxynthetic activity for L-valine, L-leucine or L-isoleucine or precursors thereof. A specific example is a L-valine producing strain of Brevibacterium lactofermentum. Mutants having high acetohydroxy acid synthase activity which showed no feedback inhibition by the end product (L-Val, L-Leu and L-Ile) can particularly be obtained by using the method described in Proceedings of the First International Congress of IAMS, 363-372p, 1975,

Haruo Momose, et al. As examples of the antagonists of these amino acids, may be mentioned α-amino-β-hydroxyvaleric acid, aminohexylisovaleric acid, isoleucine hydroxamate, 2-thiazole-alanine, β-hydroxyleucine, trichloroalanine and α-methyllysine.

The g ne for acetohydroxy acid synthase may be obtained by a process comprising, as a first step, isolating the chromosomal DNA from a DNA donor. The isolation of the chromosomal DNA can, for example, be performed by culturing a suitable DNA donor microorganism, for example a microorganism of the species Brevibacterium lactofermentum having high biosynthetic activity for L-valine, L-leucine or L-isoleucine or precursors thereof in a suitable culture medium. In a preferred embodiment, the DNA donor microorganism, for example a L-valine-producing strain of Brevibacterium lactofermentum, is inoculated in a suitable culture medium containing N-sources, C-sources and minerals, for example polypeptone, glucose and sodium chloride, over an extended period of time at normal or slightly elevated temperatures, whereafter the grown cells are collected at the stage of multiple propagation. The obtained microbial cells are lysed in a per se known manner, whereafter the chromosomal DNA is extracted and purified by methods known per se.

The obtained chromosomal DNA thereafter is properly cleaved. This can be performed in the manner also known per se by suitable restriction enzymes which are selected in accordance with the restriction site. As a specific preferred example of the present application, the chromosomal DNA obtained from a Brevibacterium lactofermentum culture is partially cleaved at the Sau3 A I cite. Fragments of 2 to 6 Kb were cut out from the chromosomal DNA.

The further step for obtaining the gene for acetohydroxy acid synthase consists of inserting the DNA fragments as obtained in the previously described steps into a suitable plasmid. Plasmids are considered to be "suitable" plasmids if they have the capability of autonomic multiplication in coryneform bacterial cells, for example cells of the genus Brevibacterium.

The subsequent step of the overall process consists of introducing the resulting recombinant plasmid into a microorganism of Brevibacterium defective in acetohydroxy acid synthase, and picking out a transformant imparted with the ability of producing acetohydroxy acid synthase.

The gene for acetohydroxy acid synthase is then inserted into a vector plasmid capable of automatic multiplication in coryneform bacterial cells, e.g. cells of Brevibacterium. The recombinant plasmid thus obtained is introduced into a receptor microorganism strain, e.g. into cells of Brevibacterium, thereby forming a transformant with improved productivity of L-valine, L-leucine or L-isoleucine.

As the gene for acetohydroxy acid synthase, may be used either a wild-type gene or a mutant-type gene. As the latter type, the most preferred is the one so mutated as to code for acetohydroxy acid synthase with reduced feedback inhibition by L-valin, L-leucine or L-isoleucine.

A mutant-type gene may be obtained by directly subjecting a DNA donor to mutation treatment. However, it can be obtained at a higher probability by inserting the gene for acetohydroxy acid synthase into a vector plasmid, introducing the recombinant DNA thus formed into host cells, and mutating the transformant thus obtained. Furthermore, the mutant-type gene can be obtained at the highest probability if the above recombinant DNA is subjected to in-vitro mutation treatment.

The mutant-type gene that codes for acetohydroxy acid synthase with reduced feedback inhibition by L-valine, L-leucine or L-isoleucine can be sorted out by selecting a DNA donor strain or a transformant imparted with resistance to antagonist of these amino (after obtaining transformants when the recombinant DNA is subjected to mutation treatment and the mutated recombinant DNA thus formed is introduced into host cells).

In order to confirm that the DNA donor strain or transformant imparted with resistance to antagonist contains a mutant-type gene that codes for acetohydroxy acid synthase with reduced feedback inhibition by L-valine, L-leucine or L-isoleucine, it is necessary to prepare an enzyme solution from that antagonist resistant strain or transformant, to allow this solution to act upon pyruvic acid (as substrate) in the presence of L-valine, L-leucine or L-isoleucine, and to measure the amount of 2-acetolactic-acid formed. An enzyme solution that shows high activity of forming 2-acetolactic acid even at a high concentration of L-valine, L-leucine or L-isoleucine is regarded as containing acetohydroxy acid synthase with reduced feedback inhibition by these amino acids.

Mutation of the DNA donor, transformant or recombinant DNA may be effected by treatment with a mutagen, such as N-methyl-N′-nitro-N-nitrosoguanidine and sulfurous acid, or by irradiation with X-rays, UV-rays or γ-rays. These methods are also efficient for mutating any genes other than that for acetohydroxy acid·synthase. In addition, these can also be applied even when the host cells are coryneform glutamate-producing bacteria other than Brevibacterium, such as Corynebacterium, or other microorganisms such as Esch richia coli, and Bacillus subtilis.

As the promotor operator for acetohydroxy acid synthase gene, may be used either a wild-typ operator

or a mutant-type operator. It is also possible to use any powerful promotors which are known to efficiently express other types of genes. The promotor operator DNA may be the one derived from other types of living bodies or the one chemically synthesized. Furthermore, the mutant-type acetohydroxy acid synthase formed in plasmid may be introduced again into the chromosome of host cells, and the same applies to th gene derived from other types of living bodies or chemically synthesized.

[Eamples]

The following examples will further illustrate the invention.

Example 1

(1) Preparation of chromosomal DNA

L-valine-producing strain of Brevibacterium lactofermentum was inoculated to 50 ml of CM2G medium (containing 10 g polypeptone, 10 g Y.E., 5 g glucose and 5 g NaCl), shake culture was continued overnight at 31°C, and the grown cells were collected at the stage of multiple propagation. One gram of the microbial cells thus obtained were lysed with lysozyme•SDS, and the chromosomal DNA was extracted and purified by the phenol method, giving 5 mg of final product.

(2) Preparation vector DNA

pAJ220 (5445 bp) was used as vector, and its DNA was prepared by the usual method.

(3) Insertion of chromosomal DNA into vector

The chromosomal DNA obtained in (1) above (30 μg) was partially cleaved at Sau3A I site, and fragments of 2 to 6 kb were further cut out. Separately, 1 μg of the vector DNA was completely cleaved with restriction endonuclease BamH I. The two types of fragments thus obtained were linked together by using Takara ligation kit.

(4) Cloning of acetohydroxy acid synthase gene

Brevibacterium lactofermentum AJ12403 (FERM P-10140, FERM BP-2507) defective in acetohydroxy acid synthase activity (a strain requiring both L-isoleucine and L-valine) was used as the receptor strain.

Transformation was carried out according to the protoplast method described in Japanese published unexamined patent no. 149082/1986, and four strains imparted with resistance to trimethoprim and rendered isoleucine- and valine- independent were obtained from the transformants.

(5) Analysis of plasmids in the transformants

Each of the four strains obtained above was lysed with lysozyme•SDS and DNA was recovered from the resulting solution.

Analysis of plasmid DNA by electrophoresis on agarose revealed that each is a plasmid larger than pAJ220 and having a DNA fragment of about 3 kb incorporated therein. One of these plasmids was named pAJ220V3.

(6) Retransformation

Brevibacterium lactofermentum AJ12403 was again transformed by plasmid pAJ220V3. All of the

transformants picked out on the basis of trimethoprim resistance were found to be independent on L-isoleucine and L-valine, indicating that the above-mentioned DNA fragm nt of about 3 kb carries the gen for acetohydroxy acid synthase.

(7) Acetohydroxy acid synthase activity of transformants

A strain being tested was cultivated in production medium (A) for about 18 hours, the grown cells were ultrasonicated, and the resulting solution was centrifuged at 15,000 r.p.m. for 20 minutes.

The supernatant thus obtained was used as a crude enzyme solution, which was added to a solution containing 65mM phosphate buffer and 100mM sodium pyruvate to effect enzymic reaction. Acetolactic acid thus formed was converted to acetoin, and the color developed by addition of 2-naphthol was measured at 530 nm. The result obtained is shown in Table 1.

It was demonstrated that cloned gene for acetohydroxy acid synthase has been incorporated in pAJ220V3.

Table 1

Strain	Amino acid added (5mM)			
	None	L-Ile	L-Leu	L-Val
AJ12403 FERM BP-2507	N.D.	—	—	—
AJ12403/pAJ220V3	100	101	119	108
ATCC13869	100	60	67	60

N.D.: Not deteced. The above figures represent relative activity values (%) when the activity in the absence of amino acid is taken as 100.

(8) Restriction enzyme cleavage map for plasmid pAJ220V3

The inserted DNA fragment of about 3 kb involves at least two sites for EcoRv and Pst I each, and one site for Bcl I, EcoR I and Bal I each. A simple restriction enzyme cleavage map illustrating these sites is shown in Figure 1.

Example 2

(1) L-valine productivity of transformant derived from wild-type strain

AJ12404 (FERM P-10141, FERM BP-2508) and AJ12405 (FERM P-10142, FERM BP-2516), obtained by transforming ATCC13869 (a wild-type strain of Brevibacterium lactofermentum) and AJ1510 (a wild-type strain of Brevibacterium flavum) respectively with pAJ200V3, showed outstanding L-valine productivity as can be seen from Table 2.

Table 2

Strain	L-valine (g/l)*
ATCC13869/pAJ220V3 (AJ12404 FERM BP-2508)	7
AJ1510/pAJ220V3 (AJ12405 FERM BP-2516)	11
ATCC13869	0
AJ1510	0

* Production medium (A): Glucose (100 g), ammonium sulfate (45 g), monopotassium phosphate (1 g), magnesium sulfate (1 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $MnSO_4 \cdot 7H_2O$ (0.01 g), Mamenou (extracted of soybean hydrolysed with HCl) (0.15 g: total nitrogen), biotin (100 µg) and vitamin B1 hydrochloride (300 µg) were dissolved in distilled water, the pH was adjusted to 7.2 by addition of KOH, water was further added to make up a total volume of one liter, and the resulting mixture was dispensed in 50-ml Sakaguchi flasks (20 ml in each) and subjected to autoclave sterilization (115°C x 10 minutes), followed by addition of dry-sterilized calcium carbonate (1 g each).

Example 3

(1) L-isoleucine productivity in the presence of threonine deaminase

Into a wild-type strain of Brevibacterium flavum AJ1510 , were introduced pAJ220V3 and pDR1A4 (a plasmid carrying threonine deaminase gene (ilvA$^+$) of Escherichia coli desensitized against L-isoleucine), giving a strain AJ12406 (FERM P-10143, FERM BP-2509) containing these two types of plasmids.

As can be seen from Table 3, AJ12406 showed increased relative activity for both threonine deaminase (TD) and acetohydroxy acid synthase (AHAS) compared with the wild-type strain. It also showed higher L-isoleucine productivity, as shown in Table 4, compared with the transformants carrying only one of the above plasmids.

Table 3

Strain	TD (nmol/min/mg)	AHAS (nmol/min/mg)
AJ1510/pDR1A4,pAJ200V3 (AJ12406, FERM BP-2509)	157.2	3.65
AJ1510/pAJ220V3 (AJ12405 FERM BP-2516)	12.8	5.11
AJ1510/pDR1A4 (AJ12358)	135.7	1.04
AJ1510	35.2	1.10

Table 4

Strain	L-isoleucine (g/l)*
AJ1510/pDR1A4,pAJ220V3 (AJ12406, FERM BP-2509)	4.6
AJ1510/pAJ220V3 (AJ12405, FERM BP-2516)	2.4
AJ1510/pDR1A4 (AJ12358)	2.5
AJ1510	0

* Production medium (A): Glucose (100 g), ammonium sulfate (50 g), monopotassium phosphate (1 g), magnesium sulfate (0.4 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $MnSO_4 \cdot 7H_2O$ (0.01 g), Mamenou (extracted of soybean hydrolysed with HCl (0.6 g; total nitrogen), biotin (50 µg) and vitamin B_1 hydrochloride (3 mg) were dissolved in distilled water, the pH was adjusted to 8.3 by addition of KOH, water was further added to make up a total volume of one liter, and the resulting mixture was dispensed in 50-ml Sakaguchi flasks (20 ml in each) and subjected to autoclave sterilization (115°C x 15 minutes), followed by addition of dry-sterilized calcium carbonate (1 g each).

Brevibacterium flavum AJ 12405, Brevibacterium flavum AJ 12406, Brevibacterium lactofermentum AJ 12403 and Brevibacterium lactofermentum AJ 12404 were originally deposited on July 20, 1988 at the Fermentation Research Institute, Agency of Industrial Sciences and Technology, Ministry of International Trade Industry (FRI), 1-3, Higashi 1-Chome, Tukuba-Shi, Ibaragi-Ken 305, Japan, and were accorded FERM P-10142, FERM P-10143, FERM P-10140 and FERM P-10141, respectively.

The microorganisms FERM P-10143, FERM P-10140 and FERM P-10141 were then converted into deposits under the Budapest Treaty on July 10, 1989, and were accorded FERM BP-2509, FERM BP-2507 and FERM BP-2508, respectively. The microorganism FERM P-10142 was also converted into deposit

under Budapest Treaty on July 12, 1989, and was accorded FERM BP-2516.

Claims

1. A plasmid or phage capable of autonomic multiplication having the gene for acetohydroxy acid synthase incorporated therein.

2. A plasmid or phage as defined in claim 1 wherein said gene for acetohydroxy acid synthase is derived from a microorganism belonging to the genus Brevibacterium.

3. A plasmid or phage as defined in claim 1 wherein said gene for acetohydroxy acid synthase is a wild-type gene.

4. A plasmid or phage as defined in claim 1 wherein said gene for acetohydroxy acid synthase is a mutated gene released from feedback inhibition.

5. A plasmid or phage as defined in claim 1 wherein said gene for acetohydroxy acid synthase is under the control of its inherent promotor or a promotor for other type of gene.

6. A plasmid or phage as defined in claim 1 wherein said gene for acetohydroxy acid synthase is under the control of its inherent promotor or a promotor for other type of gene resistant to trimethoprim.

7. A microorganism carrying a plasmid or phage as defined in any of claims 1 through 6.

8. A microorganism as defined in claim 7, said microorganism being a coryneform, glutamate-producing bacterium.

9. A microorganism as defined in any of claims 7 or 8 which microorganism belongs to the genus Brevibacterium and preferably to a species selected from Brevibacterium flavum and Brevibacterium lactofermentum.

10. A microorganism as defined in any of claims 7 to 9 which microorganism is capable of producing valine, leucine or isoleucine.

11. A microorganism as defined in any of claims 7 to 10 which microorganism is free from repression of valine, leucine or isoleucine biosynthesis.

12. A microorganism as defined in any of claims 7 to 11, said microorganism being Brevibacterium lactofermentum, preferably Brevibacterium lactofermentum FERM-P 10141 (FERM-BP 2508).

13. A microorganism as defined in any of claims 7 to 11, said microorganism being Brevibacterium flavum, preferably Brevibacterium flavum FERM-P 10142 (FERM-BP 2516) or Brevibacterium flavum FERM-P 10143 (FERM-BP 2509).

14. A microorganism as defined in any of claims 8, 10 or 11, said microorganism being Corynebacterium glutamicum.

15. A microorganism with the gene for acetohydroxy acid synthase in the plasmid transferred to the chromosome.

16. A microorganism as defined in claim 15, said microorganism being Brevibacterium lactofermentum, preferably Brevibacterium lactofermentum FERM-P 10141 (FERM-BP 2508).

17. A microorganism as defined in claim 15, said microorganism being Brevibacterium flavum, preferably Brevibacterium flavum FERM-P 10142 (FERM-BP 2516) or Brevibacterium flavum FERM-P 10143 (FERM-BP 2509).

18. A microorganism as defined in claim 15, said microorganism being Corynebacterium glutamicum.

19. A microorganism as defined in any of claims 7 to 18, wherein an additional plasmid or phage is contained which has the effect of increasing the productivity of valine, leucine or isoleucine.

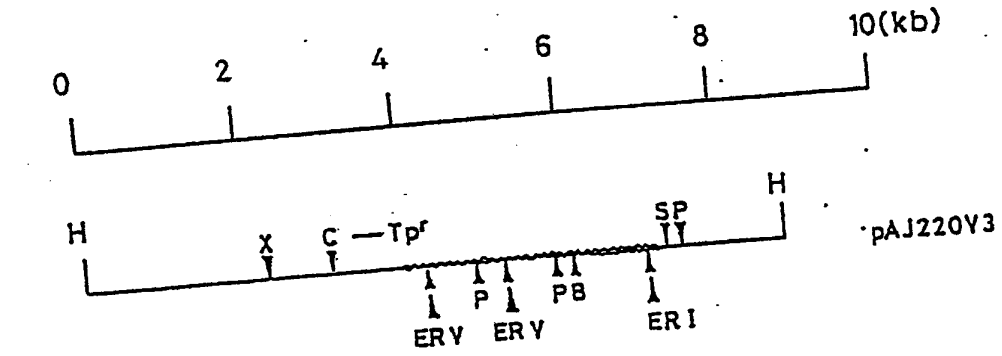
20. A microorganism as defined in claim 19, wherein the additional plasmid can coexist with the plasmid as defined in claim 1.

21. Recombinant DNA comprising the gene coding for acetohydroxy acid synthase.

22. Recombinant DNA as defined in claim 21, wherein the gene for acetohydroxy acid synthesis is obtainable from a microorganism belonging to the genus Brevibacterium having high biosynthetic activity for valine, leucine or isoleucine or precursors thereof.

23. A process for producing L-valine, L-leucine or L-isoleucine, which comprises growing a microorganism defined in any of claims 7 through 20, and recovering the objective amino acid from the culture liquor.

Fig. 1.



— pAJ220. Inserted DNA H:HindⅢ. X:XbaI. C:ClaI
S:SalI. P:PstI. ERV:EcoRV. ERI:EcoRI. B:BclI.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 89 11 4207

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	EP-A-0 183 175 (AJINOMOTO CO., INC.) * Whole document * ---	1-18,21-23	C 12 N 15/52 C 12 N 15/77 C 12 N 1/20 C 12 P 13/06 C 12 P 13/08 // (C 12 N 15/52 C 12 R 1:13 C 12 R 1:15)
Y	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 39, no. 11, November 1975, pages 2193-2198, Tokyo, JP; T. TSUCHIDA et al.: "Genetic changes of regulatory mechanisms occurred in leucine and valine producing mutants derived from Brevibacterium lactofermentum 2256" * Table 1 * ---	1-18,21-23	
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Y	DEVELOPMENTS IN INDUSTRIAL MICROBIOLOGY, vol. 24, 1983, pages 109-119, Washington, D.C., US; H. MOMOSE: "New genetic approaches to amino-acid-producing strains" * Whole article * ---	21-23	
A	BIO/TECHNOLOGY, vol. 5, no. 2, February 1987, pages 137-145, New York, US; J.F. MARTIN et al.: "Cloning systems in amino acid-producing corynebacteria" ---		TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 N C 12 P
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A	EP-A-0 233 581 (KYOWA HAKKO KOGYO CO., LTD) * Whole document * --- -/-		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10-11-1989	VAN PUTTEN A.J.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
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D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)

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EUROPEAN SEARCH REPORT

Application Number

EP 89 11 4207

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	EP-A-0 179 338 (AJINOMOTO INC.) * Claims *	19,20	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

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THE HAGUE	10-11-1989	VAN PUTTEN A.J.

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